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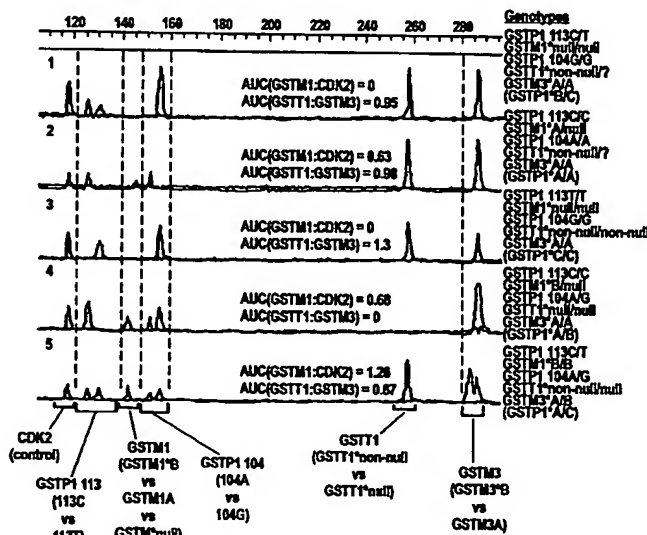
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(54) Title: HIGH-THROUGHPUT GLUTATHIONE S-TRANSFERASE POLYMORPHIC ALLELE ASSAY DESIGN



(57) Abstract: A high-throughput assay for characterizing a subject's genetic makeup is disclosed. Specifically, a high-throughput assay utilizing PCR is disclosed that permits the rapid and accurate characterization of a subject's inherited alleles of the polymorphic glutathione S-transferase (GST) genes GSTM1, GSTM3, GSTP1, and GSTT1. This method allows detection of the specific alleles inherited, including the gene dosage of GSTM1 and GSTT1 while not requiring restriction endonuclease digestion of the PCR products in order to detect length differences. Further, the method allows all analyses to be performed simultaneously in the same gel lane, thus further adding efficiency and cost-effectiveness.



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High-Throughput Glutathione S-Transferase Polymorphic Allele Assay Design**BACKGROUND OF THE INVENTION****1. Field of the Invention**

5 The present invention relates to high-throughput assays for characterizing a subject's genetic makeup. Specifically, the instant invention is a high-throughput assay that permits the rapid and accurate characterization of a subject's inherited alleles of the polymorphic glutathione S-transferase (GST) genes GSTM1, GSTM3, GSTP1, and GSTT1.

2. Background of the Invention

10 Recent cancer research has shown that the presence of various polymorphisms of glutathione S-transferase ("GST") correlates with altered risk for certain cancers and altered response and toxicity from currently known and used cancer treatments, including chemotherapy. The GST family of enzymes has been shown to function in the detoxification of a broad range of environmental and non-environmental DNA-damaging carcinogens such as polyaromatic hydrocarbons like those found in first- and second-hand cigarette smoke. Additionally, however, these enzymes are capable of detoxifying chemotherapeutic compounds such as alkylating agents and anthracyclines as well as reactive oxygen species and peroxides. See S. Tsuchida and K. Sato, *Critical Reviews in Biochemistry and Molecular Biology*, 27(4, 5):337-384 (1992), and S.A. Weitzman and L.I. Gordon, *Blood*, 76(4):655-663 (1990).

15 This family of enzymes has been subdivided into four subclasses, including GSTM1, GSTM3, GSTP1, and GSTT1. Ali-Osman *et al.*, *J. Biol. Chem.*, 272(15):10004-10012 (1997); Fryer *et al.*, *Biochem. J.*, 295:313-315 (1993); Inskip *et al.*, *Biochem. J.*, 312:713-716 (1995); and Pemble *et al.*, *Biochem. J.*, 300:271-276 (1995). A first of these is the GSTM1 class, which includes the following allelic variants: GSTM1*null, GSTM1*A, and GSTM1*B. Fryer *et al.*, *Biochem. J.*, 295:313-315 (1993).

20 GSTM1*null is thought to result from an unequal crossing-over at a duplicated region between the GSTM1 and the GSTM2 loci. Pearson *et al.*, *Am. J. Hum. Genet.*, 53:220-233 (1993); and Xu *et al.*, *J. Biol. Chem.*, 273:3517-3527

(1998). As with other null alleles, this one produces no functional product and thus acts as a recessive gene. GSTM1*A and GSTM1*B are polymorphic alleles commonly thought to result from a C to G substitution at codon 173. This change results in a change from the Lys¹⁷³ of GSTM1*A to the Asn¹⁷³ of GSTM1*B. This change alters a hinge region between alpha helices which is involved in GSTM1 dimerization.

A second subgroup of GST enzymes is dubbed the GSTM3 class, including the GSTM3*A and GSTM3*B allelic variants. These alleles are thought to result from a 3 base-pair deletion in intron 6 which generates a YY1 negative transcription factor recognition site in GSTM3*B that does not exist in GSTM3*A.

A third such subgroup of GST enzymes is the GSTP1 group which includes GSTP1*A, GSTP1*B, and GSTP1*C. A GSTP1*D allele has been observed, but only in very rare circumstances. These polymorphic alleles result from A to G and C to T transitions at nucleotides +313 of exon 5 and +341 of exon 6, respectively. Specifically, GSTP1*A codon 104 is ATC, coding for Ile¹⁰⁴, and codon 113 is GCG, for Ala¹¹³. GSTP1*B codon 104 is GTC, coding for Val¹⁰⁴, and codon 113 is GCG for Ala¹¹³. GSTP1*C codon 104 is GTC, coding for Val¹⁰⁴, and codon 113 is GTG for Val¹¹³. GSTP1*D codon 104 is ATC, coding for Ile¹⁰⁴, and codon 113 is GTG for Val¹¹³.

A fourth group of GST enzymes is the GSTT1 subgroup, which includes GSTT1*null and GSTT1. As with the other null allele noted above, the GSTT1*null allele produces no functional product, thus operating as a recessive allele.

There are techniques extant in the art for assessing which alleles are present in an individual's genotype. Most of these assays do not allow investigators to determine gene dosages. Here, the term "gene dosage" is used to denote whether one or both alleles were present when a PCR product suggested the presence of at least one non-null allele. Further, most of the currently used assays do not differentiate between the non-null GSTM1 alleles GSTM1*A and GSTM1*B. These methods also generally require that the PCR products undergo restriction endonuclease digestion to allow the determination of genotypes, thus adding extra complexity and expense to the method.

Kristensen *et al.* reported one such assay in 1998. Kristensen *et al.*, *Pharmacogenetics*, 8:441-447 (1998). This assay was able to evaluate only the polymorphisms of GSTM1, GSTP1, and GSTT1, while not being capable of

distinguishing between GSTM1*A and GSTM1*B. Further, when looking at GSTP1, the assay examined only codon 104, ignoring the polymorphisms showing changes at codon 113. Finally, as with other known assays, this assay method did not examine the gene dosage of either GSTM1 or GSTT1.

5 The availability of simple, effective assays could allow the analysis of individuals' genomes in order to detect risk for specific diseases and cancers and to allow the development of individualized prevention and/or treatment strategies. Further, in regard to the specific family of enzymes referenced above, specific, accurate assays could allow the development of tailored therapeutic regimens for patients predicted to have decreased therapeutic response to medical therapy, including cancer therapy, based on their expression of GST enzymes, or for patients predicted to have increased therapy-related toxicity. Additionally, such assays would simplify the implementation of patient-specific utilization of allele-specific small-molecule inhibitors for the purpose of reversing chemotherapy resistance among cancers, such as those over-expressing certain GST polymorphic alleles.

10 From the above, it is apparent that it would be an improvement in the art to provide a high-throughput assay method for rapidly, inexpensively, and accurately characterizing the GST alleles present in a subject. It would be a further advancement in the art to provide a high-throughput GST assay method which is capable of accurately determining the gene dosage of GSTM1 and GSTT1 using competitive PCR. Additionally, it would be a further advancement in the art to provide such an assay design which is simpler, faster, and cheaper than those currently known in the art because it does not require restriction endonuclease digestion of PCR products in order to elucidate the length differences between GST alleles. Similarly, it would be an improvement in the art to allow the assay of all of the PCR products simultaneously in a single gel lane, which would yield further savings in time and expense. Finally, it would be an improvement in the art to provide a high-throughput assay method that would comprehensively assay all four GST polymorphs and their alleles, including null alleles. Such an assay method is disclosed herein.

SUMMARY OF THE INVENTION

25 The present invention provides methods and apparatus for detecting the presence of glutathione S-transferase alleles using PCR methods and unique primers

in such a way as to allow accurate genotyping by detecting the alleles of GSTM1, GSTM3, GSTT1, and GSTP1 present in a sample, while also allowing the detection of the gene dosage of GSTM1 and GSTT1.

5 This invention is important to the feasibility and future success of large population studies of how differences in the genotype of the alleles of glutathione S-transferase correlate with risk for cancer, as well as how the alleles correlate with possibility of risk and successful outcomes of therapy for a group of diseases including cancer. Specifically, GST enzyme polymorphisms have been correlated with risks for cancer and with risks of altered response and toxicity from cancer
10 treatments. Existing assays for the alleles of GSTM1, GSTM3, GSTT1, and GSTP1 require multiple PCR runs followed by endonuclease digestion of the PCR products in order to detect the length differences between the GST polymorphic alleles. Most currently known and practiced assays are incapable of demonstrating the presence of the alleles of all four polymorphs. Further, both GSTM1 and GSTT1 have null alleles,
15 and existing assays cannot show the presence of these alleles or the corresponding gene dosage of GSTM1 and GSTT1. Finally, current analysis methods are very expensive. This fact places strong restrictive limits on the size of the population evaluated in many recent studies.

The instant invention overcomes these limitations by using carefully designed
20 PCR primers in paired PCR competitions to allow each of the alleles of the polymorphisms of GST to be detected, including both null alleles. Table 1 shows one set of possible primers that can be used with the instant invention. Forward primers are indicated by "Fwd," and reverse primers are indicated by "Rvs." TET and FAM are fluorescent tags that can be used to detect sequences by automated
25 polyacrlamide gel electrophoresis. Boxed sequences indicate non-sequence specific tails used to create PCR product length polymorphisms. Underlined nucleotides indicate single nucleotide polymorphisms.

Table 1. High Throughput Genotyping: PCR Primers

Gene	Primer name	Fwd /Rvs	Primer Sequence	PCR Product
GSTM1	M1F-A	Fwd	5'-(TET)TTGGGAAGGCG TCCAAGCAC-3'	142 bp (GSTM1*A)
	M1F-B	Fwd	5'-(FAM)TCITTGGGAA GGCGTCCAAGCAG-3'	145 bp (GSTM1*B)
	M1R	Rvs	5'-GTTTCTTCTGCTTCAC GTGTTATGAAGGTT-3'	0 bp (GSTM1*null)
GSTM3	M3F	Fwd	5'-(FAM)GTTTCTCCTCA GTACTTGAAGAGCT-3'	287 bp (GSTM3*A)
	M3R	Rvs	5'-GTTTCTCACATGAAA GCCTTCAGGTT-3'	284 bp (GSTM3*B)
GSTP1	P1-104FA	Fwd	5'-(FAM)GTTTCTGACCTC CGCTGCAAATACA-3'	150 bp (Ile ¹⁰⁴)
	P1-104FG	Fwd	5'-(TET)GTTTCTCTTGAC CTCCGCTGCAAATACG-3'	153 bp (Val ¹⁰⁴)
	P1-104R	Rvs	5'-GTTTCTCAGCCCAAGC CACCTGA-3'	
	P1-113FC	Fwd	5'-(TET)CTTTGGTGTCTG GCAGGAGGT-3'	130 bp (Ala ¹¹³)
	P1-113FT	Fwd	5'-(FAM)GGTGTCTGGCA GGAGGC-3'	126bp(Val ¹¹³)
	P1-113R	Rvs	5'-GTTTCTTGGTCTCCCA CAATGAAGG-3'	
GSTT1	T1F	Fwd	5'-(FAM)TTCCTTACTGGT CCTCACATCTC-3'	255bp (GSTT1)
	T1R	Rvs	5'-GTTTCTACAGACTGG GGATGGATGGTT-3'	0 bp (GSTT1*null)

5 In one embodiment, the invention is a method including the steps of obtaining a sample of genomic DNA and conducting three separate PCR runs of portions of the sample. After this, the alleles may be detected. In one embodiment of the invention, a first portion of the sample is amplified by PCR with primers for GSTM1 and CDK2; a second portion is amplified with primers for GSTT1 and GSTM3; and a third portion

10 of the sample is amplified with primers for GSTP1. Following this, the alleles may be detected. In the above method, at least one of the primers used further has a fluorescent such as TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX (Applied Biosystems, Foster City, CA). Further, the PCR products of the

amplification of the first, second, and third portions of the sample are combined prior to the step of detecting the presence and the gene dosage of the alleles. This detection step may be accomplished by conducting a gel electrophoresis of the sample using a single lane. This allows cost savings over traditional methods. The detection step may next involve fluorescence detection to determine the presence of PCR products for the specific alleles. In addition, the detection step may further involve comparing the areas under the peak of GSTM1*A or GSTM1*B PCR products with the area under the peak of the CDK2 PCR products to determine the gene dosage of GSTM1; as well as comparing the areas under the peak of the GSTM3*A or GSTM3*B PCR products with the area under the peak of the GSTT1 PCR products in order to determine the gene dosage of GSTT1.

The detection step noted above may be varied significantly within the scope of the instant invention. In some forms of the invention, capillary electrophoresis is used in the place of typical gel electrophoresis, followed by fluorescence detection to determine the presence of PCR products for the specific alleles and comparison of the areas under the peak of the GSTM1*A or GSTM1*B PCR products with the area under the peak of the CDK2 PCR products to determine the gene dosage of GSTM1 and comparison of the areas under the peak of the GSTM3*A or GSTM3*B PCR products with the area under the peak of the GSTT1 PCR products to determine the gene dosage of GSTT1.

In other forms of the method of the instant invention, the primers include internally-biotinylated nucleotides. In these methods, the step of detecting the presence and gene dosage of the alleles comprises the steps of combining the PCR products of the amplification of the first, second, and third portions of the sample; exposing the mixture to streptavidin-coated magnetic or non-magnetic beads; and detecting the presence or absence of allele-specific PCR products through the use of a microplate reader capable of indicating the presence or absence of allele-specific PCR products.

Other forms of the invention utilize real-time PCR to amplify and detect the alleles of GSTM1, GSTM3, GSTT1, and GSTP1. This method is useful due to its relative ease and simplicity and its ability to amplify and detect the alleles and determine the gene dosages of GSTM1 and GSTT1 alleles all at the same time and without using specialized equipment, excepting the real time PCR machine itself.

In other forms, the method includes primers that include at least one radiolabeled nucleotide and uses methods known in the art for detecting the radiolabeled molecules, including electrophoresis followed by autoradiography or phosphoimaging and related techniques.

5 In other embodiments of the invention, it is a high-throughput assay for the detection of glutathione S-transferase polymorphic alleles comprising the steps of: obtaining a sample of genomic DNA; conducting PCR amplification of the genomic DNA using primers for GSTM1, CDK2, GSTT1, GSTM3, and GSTP1; combining the PCR reaction products into one mixture; and detecting the presence and the gene
10 dosage of the alleles. Here, the primers may further include labels such as: fluorescent nucleotide dyes (TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX)(Applied Biosystems, Foster City, CA), biotinylated deoxynucleotides, radioactive phosphorus deoxynucleotides, or radioactive sulfur phosphorothioates, and others that could function properly in this application. The detection step also
15 involves loading the mixture into a single gel lane and conducting gel electrophoresis or capillary electrophoresis.

A presently preferred embodiment of the instant invention is a method of identifying the alleles of glutathione S-transferase present in a sample of genetic material which includes three distinct PCR steps which may be performed in any
20 order. A first step is to conduct the PCR amplification of the GSTM1 alleles and CDK2 present in a first portion of a genetic sample using primers comprising polynucleotide sequences substantially identical to SEQ ID NO: 1, nucleotides 3-23 of SEQ ID NO: 2, nucleotides 9-31 of SEQ ID NO: 3, SEQ ID NO: 14, and SEQ ID NO: 15; wherein at least one of said primers further comprises a signal marker. A
25 second step is to conduct the PCR amplification of the GSTT1 and GSTM3 alleles present in a second portion of the sample using primers comprising polynucleotide sequences substantially identical to nucleotides 7-26 of SEQ ID NO: 4, nucleotides 7-26 of SEQ ID NO: 5, SEQ ID NO: 12, and nucleotides 6-27 of SEQ ID NO: 13; wherein at least one of said primers further comprises a signal marker. A third step is
30 to conduct the PCR amplification of the GSTP1 alleles present in a third portion of the sample using primers comprising polynucleotide sequences substantially identical to nucleotides 7-25 of SEQ ID NO: 6, nucleotides 10-28 of SEQ ID NO: 7, nucleotides 6-23 of SEQ ID NO: 8, nucleotides 4-21 of SEQ ID NO: 9, SEQ ID NO:

10, and nucleotides 7–25 of SEQ ID NO: 11; wherein at least one of said primers further comprises a signal marker. The detection step follows these amplification steps, and shows the presence or absence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1.

5 As with other embodiments, the signal marker of those primers which have markers such as fluorescent dyes (TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX) (Applied Biosystems, Foster City, CA), biotinylated deoxynucleotides, radioactive phosphorus deoxynucleotides, or radioactive sulfur phosphorothioates, and may include other suitable markers. Also, in some of the
10 methods, the PCR products of the amplification steps are combined prior to the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1. Further, this step may simply comprise analyzing the allele makeup and gene dosage of the sample using a single lane of a gel electrophoresis. This step could also be detecting the presence of the alleles of
15 GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1 using capillary electrophoresis.

In other forms of the instant invention, the primers may additionally comprise internally-biotinylated nucleotides. In these, the step of detecting the presence and gene dosage of the alleles includes the steps of combining the PCR products of the
20 amplification of the first, second, and third portions of the sample; exposing the resulting mixture to streptavidin-coated magnetic or non-magnetic beads; and detecting the presence or absence of allele-specific PCR products through the use of a microplate reader capable of indicating the presence or absence of allele-specific PCR products.

25 In still other forms of the invention, the steps of amplifying a first portion of the sample with primers for GSTM1 and CDK2; amplifying a second portion of the sample with primers for GSTT1 and GSTM3; amplifying a third portion of the sample with primers for GSTP1; and detecting the presence and the gene dosage of the alleles are accomplished using real-time PCR, wherein amplification and detection of alleles
30 is conducted simultaneously. In some of these embodiments, the signal markers comprise radiolabeled nucleotides. In these, the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1 comprises detecting the radiolabeled PCR products.

Further, in some embodiments, the instant invention is a novel PCR primer comprising a nucleotide sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13. In some of these, the PCR primer further includes a signal marker selected such as fluorescent dyes (TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX)(Applied Biosystems, Foster City, CA). These primers may further comprise radiolabeled nucleotides and/or internally-biotinylated nucleotides and/or nucleotide analogs within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWING

In order that the invention may be more readily understood, a more particular description of the invention briefly described above will be rendered by reference to specific embodiments thereof which are illustrated in the appended drawing. Understanding that this drawing depicts only typical embodiments of the invention and are not therefore to be considered to be limiting of its scope, the invention will be described and explained with additional specificity and detail through the use of the accompanying drawings in which:

Figure 1 shows the result of the high-throughput GST polymorphic allele assay of the instant invention for five DNA samples taken from five different human cell lines.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Polymorphisms of glutathione S-transferase (GST) enzymes have been correlated with clinically significant changes of risk for many different cancers. Specifically, cancer risk differences have been associated with polymorphic alleles of GSTM1, GSTM3, GSTP1, and GSTT1. See e.g., Krajinovic *et al.*, *Blood*, 93:1496–1501 (1999), Jahnke *et al.*, *Am. J. Surg.*, 172:671–673 (1996), Volm *et al.*, *Med. & Ped. Onc.*, 28:117–126 (1997), and Elexpuru-Camiruaga *et al.*, *Canc. Res.*, 55:4237–4239 (1995). Differences in response, toxicity, and outcome of treatment for cancer have been associated with polymorphic alleles of GSTM1 and GSTP1. See e.g., Goto *et al.*, *Canc. Res.* 56:3725–3730 (1996), and Volm *et al.*, *Med. & Ped. Onc.*, 28:117–126 (1997). These and other studies conducted on this topic are retrospective studies,

however, and thus prospective examinations of risk and response through national trials should lead to the discovery of better-defined relationships between the possible GST genotypes and cancer and cancer treatment success levels.

5 GST polymorphic alleles occur at frequencies of at least 2.4–20%, and may be as high as 40–84%. This fact alone renders them socially and medically important. The frequencies of these alleles vary among diverse ethnic groups, as seen in Tables 2, 3, 4, and 5 below. Table 2 contains statistics regarding the GSTM1 polymorphic allele frequency in diverse ethnic groups. The number of patients examined in each ethnic group is designated as “n”. GSTM1*null is designated as “null.” GSTM1*A and GSTM1*B are designated as M1*A and M1*B respectively.1

Table 2. GSTM1 Polymorphic Allele Frequencies among Diverse Ethnic Groups

Ethnic Group	(n)	Null: null	Null: M1*A; Null: M1*B; M1*A/M1*B; or M1*A/M1*B
Multinational	253	50	50
Washington Co., MD (caucasians)	110	64.5	35.5
African-Americans	59	40.7	59.3
Quebec, Canada	174	64.9	35.1
United Kingdom	84	44	66
United Kingdom	300	57.3	42.7
England	113	63.7	56.3
Finland	142	43.7	56.3
Berlin, Germany	400	50.7	49.3
Parma, Italy	98	50	50
Linxian County, China	45	53	47
Taiwan	150	63.3	36.7

15 Table 3 shows GSTM3 polymorphic allele frequencies in two populations from Great Britain. The number of patients examined in each population is designated as “n”. M3*A indicates a GSTM3*A allele, and M3*B indicates a GSTM3*B allele.

Table 3. GSTM3 Polymorphic Allele Frequencies

Ethnic Group	(n)	M3*A	M3*B	M3*A: M3*A	M3*A: M3*B	M3*B: M3*B
Great Britain	244	84.2	15.8	70.9	25.8	3.4
Great Britain	300			73.7	21.3	5.0

Table 4 shows GSTP1 polymorphic allele frequencies among diverse ethnic groups from all over the world. The number of patients examined in each ethnic group is designated as "n". P1*A, P1*B, and P1*C indicate GSTP1*A GSTP1*B GSTP1*C alleles respectively.

Table 4. GSTP1 Polymorphic Allele Frequencies among Diverse Ethnic Groups

Ethnic Group	(n)	P1*A	P1*B	P1*B or P1*C	P1*C
African-American	112	56.6-58%	39.9%		2.1-5%
European	199	61.4%	31.6%		2.4%
Scottish	155	72.3%		27.7%	
Norwegian	297	71.2%		28.8%	
German Caucasian	180	69.5%		30.5%	
Indian	40	66.4%	24.6%		2.4%
Chinese	49	80.2%	18.8%		0.2%
Linxian County, China	36	76.5%		23.5%	
Japanese	164	83.5%		16.5%	
Aboriginese	45	89%	11%		0%

Finally, Table 5 shows GSTT1 polymorphic allele frequencies among diverse ethnic groups from all over the world. The number of patients examined in each ethnic group is designated as "n". GSTT1*null is indicated by "null." "T1" indicates a GSTT1 allele.

Table 5. GSTT1 Polymorphic Allele Frequencies among Diverse Ethnic Groups

Ethnic Group	(n)	Null:null	Null:T1 or T1:T1
USA	190	16	84
Washington Co., MD	110	27.2	72.7
African-American	119	21.8	78.2
African-Americans	59	28.8	71.2
Mexican-American	73	9.7	90.3
French Canadian	176	15.9	84.1
United Kingdom	494	18.4	81.6
Sweden	270	9.6	90.4
Australia	94	19	81
Chinese	45	64.4	35.6
Linxian County, China	45	49	51
Korean	103	60.2	39.8
Japanese	126	44.4	55.6

The assay design of the instant invention has four advantages over existing high-throughput assays. Because most GST genotyping assays currently known in the art are solely PCR-based, it has not been possible for investigators to determine gene dosage in the course of the assay. Gene dosage denotes an evaluation of whether one or both alleles were present when a PCR product suggested the presence in the genotype of at least one non-null allele. Further, these assays generally do not differentiate between the non-null GSTM1 alleles (GSTM1*A and GSTM1*B). Most of these examinations further require the additional step of digesting the PCR products with restriction endonucleases in order to determine the genotype.

A first step in the method is to assay for the three polymorphic alleles of GSTM1. The GSTM1 class includes the GSTM1*null, GSTM1*A, and GSTM1*B allelic variants. Fryer *et al.*, *Biochem. J.*, 295:313–315 (1993). GSTM1*null is thought to result from an unequal crossing-over at a duplicated region between the GSTM1 and the GSTM2 loci. Pearson *et al.*, *Am. J. Hum. Genet.*, 53:220–233 (1993); and Xu *et al.*, *J. Biol. Chem.*, 273:3517–3527 (1998). As with other null alleles, this one produces no usable product and thus exists as a functional genetic recessive. GSTM1*A and GSTM1*B are polymorphic alleles commonly thought to result from a C to G substitution at codon 173. This change results in a change from the Lys¹⁷³ of GSTM1*A to the Asn¹⁷³ of GSTM1*B. This change alters a hinge region between alpha helices which is involved in GSTM1 dimerization. The polymorphic alleles of Glutathione S-OTransferase Genes are summarized in Table 6.

Table 6. Polymorphic Alleles of Glutathione S-Transferase Genes

Class/ Subclass	Locus	Allelic Variants	Codon	Significance
Mu <u>GSTM1</u>	1p13.3	GSTM1*null GSTM1*A GSTM1*B	Lys ¹⁷³ Asn ¹⁷³	- Absent Allele - Hinge between to alpha helixes at dimerization site
<u>GSTM3</u>	1P13.3	GSTM3*A GSTM3*B	Full Intron 6 3bp Deletion in Intron 6	- Unknown; the deletion generates a YY1 Negative Transcription Factor Recognition Site
Pi <u>GSTP1</u>	11q13	GSTP1*A GSTP1*B GSTP1*C	Ile ¹⁰⁴ (104a), Ala ¹¹³ (113C) Val ¹⁰⁴ (104G), Ala ¹¹³ (113C) Val ¹⁰⁴ (104G), Val ¹¹³ (113T)	- Modified contacts at the binding site for electrophilic carcinogens (H- site)
Theta <u>GSTT1</u>	22q11	GSTT1*null GSTT1*T1		- Absent Allele

Possible GSTM1 genotypes are GSTM1*A/GSTM1*A, GSTM1*A/GSTM1*B, GSTM1*B/GSTM1*B, GSTM1*A/GSTM1*null, GSTM1*B/GSTM1*null, and GSTM1*null/GSTM1*null.

In the assay method of the instant invention, GSTM1 alleles can be detected by fluorescent, allele-specific PCR using two forward primers, M1FA (SEQ ID NO: 1) and M1FB (SEQ ID NO: 2), and one reverse primer, M1R (SEQ ID NO: 3). The sequences of these primers are as follows:

M1FA: 5'-(TET)TTGGGAAGGCGTCCAAGCAC-3'

M1FB: 5'-(FAM)TCCTTGGGAAGGCGTCCAAGCAG-3'

M1R: 5'-GTTTCTCTGCTTCACGTGTTATGAAGGTTTC-3'

The polymorphic nucleotides in the primers are placed on the 3' side of the forward primers in order to increase sequence specificity of PCR amplification. TET and FAM represent green and blue fluorescent tags, respectively. The boxed sequence of M1FB represents a non-sequence specific tail added to create PCR product length differences between the 142 base pair TET-tagged GSTM1*A PCR product and the 145 base pair FAM-tagged GSTM1*B PCR product. The boxed sequence of the reverse primer was added to prevent spectral overlap between TET- and FAM-tagged PCR products.

Both the length of the non-sequence specific tails and the tags may be easily varied within the scope of the instant invention.

CDK2 is co-amplified as a positive control for GSTM1*null/GSTM1*null genotypes and to determine GSTM1 gene dosage. CDK2 forward and reverse primers are CDK2F (SEQ ID NO: 14) and CDK2R (SEQ ID NO: 15), respectively. Sequences of CDK2F and CDK2R are as follows:

CDK2F: 5'-CCTATTCCCTGGAGATTCTG

CDK2R: 5'-(FAM)AAACTTGGCTTGTAATCAGGC

To determine gene dosage of GSTM1*A or GSTM1*B by competitive PCR, peak areas of GSTM1*A or GSTM1*B, and CDK2 PCR products can be quantified by polyacrylamide gel electrophoresis and fluorescence detection using an ABI PRISM® 373 Sequence Detection System (Applied Biosystems, Foster City, CA). Peak areas of GSTM1*A or GSTM1*B products can then be compared to the peak area of the CDK2 PCR product in order to determine GSTM1 gene dosage, thereby differentiating between the genotypes GSTM1*A/GSTM1*A and GSTM1*B/GSTM1*B and the genotypes GSTM1*A/GSTM1*null or GSTM1*B/GSTM1*null.

This step may be carried out under the following reaction or other suitable conditions: CDK2F 0.25 µM, M1R 0.5 µM, Taq Platinum Polymerase 0.25 U (Life Technologies, Rockville, MD), spermidine HCl 0.2 mM, MgCl₂ 1.5mM, NaCl 40mM, Tris-HCl (pH8.3) 10 mM, dNTPs 200 µM each. Reaction volume is 20 µl. Samples are amplified in an MJResearch PTC250 Thermacycler (MJResearch, Inc., Watertown, MA) or other suitable equipment. The amplification parameters can be as follows: denaturing for 5 minutes at 94 C, 25 temperature cycles (comprising 20 seconds at 94 C, 20 seconds at 52 C, and 40 seconds at 72 C), followed by a final extension for 10 minutes at 72 C.

A next step in the assay method is assaying for the polymorphic alleles of GSTM3. This subgroup includes the GSTM3*A and GSTM3*B allelic variants. These alleles are thought to result from a 3 base-pair deletion in intron 6 which generates a YY1 negative transcription factor recognition site in GSTM3*B that does not exist in GSTM3*A. Possible genotypes include GSTM3*A/GSTM3*A, GSTM3*A/GSTM3*B, and GSTM3*B/GSTM3*B.

In the assay of the instant invention, GSTM3 alleles may be detected by fluorescent, allele-specific PCR using one forward primer, M3F (SEQ ID NO: 4) and one reverse primer, M3R (SEQ ID NO: 5). The sequences for these primers are as follows:

5 M3F: 5'-(FAM)GTTTCTCCTCAGTACTTGGAAGAGCT-3'

M3R: 5'-GTTTCTCACATGAAAGCCTTCAGGTT-3'

The three base pair deletion for GSTM3*B is located internal to the primers M3F and M3R. The boxed sequences represent non-sequence specific tails added to adjust PCR product length. Amplification of GSTM3*A results in a 287 base pair PCR product.

10 This amplification step is compatible with, and thus may be performed concurrently with, GSTT1 PCR amplification. One suitable set of reaction conditions follow: T1F 0.5 μ M, T1R 0.5 μ M, M3FA 0.5 μ M, M3FB 0.5 μ M, M3R 0.5 μ M, Taq Platinum Polymerase 0.25 U (Life Technologies, Rockville, MD), spermidine HCl 0.2 mM, MgCl₂ 1.5 mM, NaCl 40 mM, Tris-HCl (pH 8.3) 10 mM, and dNTPs 200 μ M
15 each. Reaction volume is 20 μ l. Samples are amplified in an MJResearch PTC250 Thermacycler (MJResearch Inc., Watertown, MA) or other suitable equipment. The amplification may occur under the following sample conditions: denaturing for 5 minutes at 94 C, 25 temperature cycles (20 seconds at 94 C, 20 seconds at 58 C, 40 seconds at 72 C), followed by a final extension for 10 minutes at 72 C. Other suitable
20 amplification reaction systems will be understood by one of skill in the art.

A third step is the detection of the polymorphic alleles of GSTP1. These include GSTP1*A, GSTP1*B, GSTP1*C, and GSTP1*D. The GSTP1*D allele has been observed, but only in very rare circumstances in two individuals. Watson *et al.*, *Carcinogenesis*, 19:275-280 (1998). The GSTP1 polymorphic alleles are thought to
25 result from A to G and C to T transitions at nucleotides +313 of exon 5 and +341 of exon 6. These transitions result in ATC (Ile¹⁰⁴) to GTC (Val¹⁰⁴) and GCG (Ala¹¹³) to GTG (Val¹¹³). Specifically, GSTP1*A codon 104 is ATC, coding for Ile¹⁰⁴, and codon 113 is GCG, for Ala¹¹³. GSTP1*B codon 104 is GTC, coding for Val¹⁰⁴, and codon 113 is GCG for Ala¹¹³. GSTP1*C codon 104 is GTC, coding for Val¹⁰⁴, and codon
30 113 is GTG for Val¹¹³. GSTP1*D codon 104 is ATC, coding for Ile¹⁰⁴, and codon 113 is GTG for Val¹¹³. GSTP1 genotypes expected to be common include

GSTP1*A/GSTP1*A, GSTP1*A/GSTP1*B, GSTP1*A/GSTP1*C,
GSTP1*B/GSTP1*B, GSTP1*B/GSTP1*C, GSTP1*C/GSTP1*C.

The assay of the instant invention detects polymorphisms at codon 104 of GSTP1 by fluorescent, allele-specific PCR using two forward primers, P1-104FA (SEQ ID NO: 6) and P1-104FG (SEQ ID NO: 7), and one reverse primer, P1-104R (SEQ ID NO: 8). The sequences for these primers are as follows:

P1-104FA: 5'-(FAM)GTTTCTGACCTCCGCTGCAAATACA-3'

P1-104FG: 5'-(TET)GTTTCTCTTGACCTCCGCTGCAAATACG-3'

P1-104R: 5'-GTTTCTCAGCCCAAGCCACCTGA-3'

As noted with GSTM1 above, the underlined polymorphic nucleotides are placed at the 3' side of the forward primers in order to increase sequence specificity of PCR amplification. The boxed sequences represent non-sequence specific tails added to adjust PCR product length and to create differences in length between PCR products containing an A in codon 104, (thus producing a 150 base pair PCR product) and products containing a G in codon 104 (thus producing a 153 base pair PCR product). Both the length of the non-sequence specific tails and the tags may be easily varied within the scope of the instant invention.

The reaction for this assay at codon 104 may proceed as follows or under other suitable reaction conditions: P1-104FA at 0.25 μ M, P1-104FG 0.125 μ M, P1-104R 0.5 μ M, Taq Platinum Polymerase 0.25 U (Life Technologies, Rockville, MD); spermidine HCl 0.2 mM, MgCl₂ 1.5 mM, NaCl 40 mM, Tris-HCl (pH 8.3), 10 mM, dNTPs 200 μ M each. The reaction volume is 20 μ l. Samples can be amplified in an MJResearch PTC250 Thermacycler (MJResearch Inc., Watertown, MA) or with other suitable equipment. The amplification may occur under the following conditions: denaturing for 5 minutes at 94 C, 25 temperature cycles (20 seconds at 94 C, 20 seconds at 64 C, 40 seconds at 72 C), followed by a final extension for 10 minutes at 72 C.

Polymorphisms of codon 113 of GSTP1 are similarly detected by fluorescent, allele-specific PCR using two forward primers, P1-113FC (SEQ ID NO: 9) and P1-113FT (SEQ ID NO: 10), and one reverse primer, P1-113R (SEQ ID NO: 11). The sequences for these primers are as follows:

P1-113FC: 5'-(TET)CTTTGGTGTCTGGCAGGAGGT-3'

P1-113FT: 5'-(FAM)GGTGTCTGGCAGGAGGC-3'

P1-113R: 5'-GTTTCTTGGTCTCCCACAATGAAGG-3'

The underlined polymorphic nucleotides are placed at the 3' side of the forward primers in order to increase sequence specificity of PCR amplification. The boxed sequences represent non-sequence specific tails added to adjust PCR product length and to create differences in length between PCR products containing a C in codon 113 (producing a 130 base pair PCR product), and those containing a T in codon 113 (producing a 126 base pair PCR product). Both the length of the non-sequence specific tails and the tags may be easily varied within the scope of the instant invention.

One suitable set of reaction conditions for this step are as follows: P1-113FC 0.25 μ M, P1-113FT 0.125 μ M, P1-113R 0.5 μ M, Taq Platinum Polymerase 0.25 U (Life Technologies, Rockville, MD), spermidine HCl 0.2 mM, $MgCl_2$ 1.5 mM, NaCl 40 mM, Tris-HCl (pH 8.3) 10 mM, dNTPs 200 μ M each. Reaction volume is 20 μ l. Samples may be amplified in an MJResearch PTC250 Thermacycler (MJResearch Inc., Watertown, MA) or other suitable equipment. The amplification may occur under the following reaction conditions: denaturing for 5 minutes at 94 C, 25 temperature cycles (20 seconds at 94 C, 20 seconds at 64 C, 40 seconds at 72 C), followed by a final extension for 10 minutes at 72 C.

A final step in the first portion of the assay of the instant invention is to detect the polymorphic alleles of GSTT1, including GSTT1*null and GSTT1. As with the other null allele noted above, the GSTT1*null allele produces no functional product, thus operating as a recessive allele. Possible genotypes include GSTT1*T1/GSTT1*T1, GSTT1*T1/GSTT1*null, and GSTT1*null/GSTT1*null.

GSTT1 alleles are detected by fluorescent, allele-specific PCR using the forward primer T1F (SEQ ID NO: 12), and T1R (SEQ ID NO: 13). The sequences of these primers are as follows:

T1F: 5'-(FAM)TTCCTTACTGGTCCTCACATCTC-3'

T1R: 5'-GTTTCTTACAGACTGGGGATGGATGGTT-3'

The boxed sequence of T1R represents a non-sequence specific tail added to adjust PCR product length. GSTT1 is amplified with GSTM3 as a positive control for GSTT1*null/GSTT1*null genotypes and to determine GSTT1 gene dosage. GSTM3

forward and reverse primers were used as specified above. Both the length of the non-sequence specific tails and the tags may be easily varied within the scope of the instant invention.

To determine gene dosage of GSTT1 by competitive PCR, peak areas of
5 GSTM3*A or GSTM3*B, and GSTT1 PCR products are quantified by
polyacrylamide gel electrophoresis and fluorescence detection using an ABI PRISM®
373 Sequence Detection System (Applied Biosystems, Foster City, CA). Peak areas of
GSTM3*A or GSTM3*B products are compared to the peak area of the GSTT1 PCR
product in order to determine GSTT1 gene dosage, thereby differentiating between
10 the genotypes GSTT1*T1/GSTT1*T1 and GSTT1*null/GSTT1*null.

Sample reaction conditions that can be used are as follows: T1F 0.5 µM, T1R
0.5 µM, M3FA 0.5 µM, M3R 0.5 µM, Taq Platinum Polymerase 0.25 U (Life
Technologies, Rockville, MD), spermidine HCl 0.2 mM, MgCl₂ 1.5 mM, NaCl 40
mM, Tris-HCl (pH 8.3) 10mM, dNTPs 200 µM each. Reaction volume is 20 µl.
15 Samples are amplified in an MJResearch PTC250 Thermacycler (MJResearch Inc.,
Watertown, MA) or other suitable equipment. A suitable set of reaction conditions
for the PCT amplification are as follows:: denaturing for 5 minutes at 94 C, 25
temperature cycles (20 seconds at 94 C, 20 seconds at 58 C, 40 seconds at 72 C),
followed by a final extension for 10 minutes at 72 C.

20 A final step of the instant assay is to combine the PCR products of each of the
PCR reactions above. Following this, the PCR reactants may then be loaded into a
single lane on an ABI PRISM® 373 Sequence Detection System (Applied
Biosystems, Foster City, CA). For this study, interpretation was performed manually
although automated determination of GST genotypes is possible with ABI PRISM®
25 software.

Referring to Figure 1, the results of a high throughput genotyping conducted
according to the method of the present invention is shown. The rows 1-5 each
represent DNA samples from different human cell lines. The genotype of each DNA
sample was determined by the differential length of PCT products (increasing from
30 left to right). Alleles are represented from left to right in the following order: CDK2
(a control), GSTP1 113C, GSTP1 113T, GSTM1*B, GSTM1*A, GSTM1*null,
GSTP1 104A, GSTP1 104G, GSTT1*non-null, GSTT1*NULL, GSTM3B, AND

GSTM3*A. The GSTP1 genotype designations GSTP1*A, GSTP1*B, and GSTP1*C were determined by the combination of GSTP1 113 AND GSTP1 104 genotypes as follows: GSTP1*A = 113C/104A, GSTP1*B = 113C/104G, and GSTP1*C = 113T/104G. Gene dosage for GSTM1 AND GSTT1 was determined by comparing the area under the curve (AUC) of their products to the AUC of co-amplified homozygous controls CDK2 and GSTM3, respectively. AUC ratios are near linear, but may require optimization with the first set of patient samples.

Specifically, the genotype results are shown for five separate cell-line-derived DNA samples. Each line represents four different, combined PCR reactions from a single DNA sample source. The genotype corresponding to each sample is shown at the end of each line. The gene dosage for GSTM1 was determined by examining the ratios of the peak area under the curve of GSTM1 vs. CDK2. The gene dosage for GSTT1 was determined by examining the ratios of the peak area under the curve of GSTT1 vs. GSTM3. Ratios greater than 1.0 were interpreted to represent genotypes GSTM1*non-null/GSTT1*null. Independent experiments verifying GSTM1 and GSTT1 gene dosage by an independent long range PCR assay.

Though the data in Figure 1 was gathered using DNA gathered from tissue-culture cell lines, the assay method has been shown successful in assaying DNA derived from patient-derived peripheral leukocytes. It is further useful in analyzing DNA isolated from buccal epithelial cells (taken in some instances from mouthwash samples) as well as dried blood spots taken from Guthrie cards. This characteristic of sample source versatility renders the assay method of the instant review highly useful for large clinical trials.

Finally, the design and cost of the high-throughput assay method of the instant invention render it attractive to researchers. More specifically, the methodology of the instant invention allows the determination of polymorphic alleles of four different GST genes for 96 patient samples within a period of about 10 hours at a current cost of approximately \$7.50 per sample.

As a result of these characteristics of the instant invention, it is apparent that the method of the instant invention lends itself to a wide array of applications. These include the pharmacogenetic applications of: detecting individuals at risk for specific diseases in order to aid in the development of prevention strategies, tailoring therapeutic regimens for patients predicted to have decreased therapeutic response to

medical therapy, tailoring therapeutic regimens for patients predicted to have increased therapy-related toxicity, and allowing for the patient-specific utilization of allele-specific small-molecule inhibitors for the purpose of reversing chemotherapy resistance among cancers over-expressing certain GST polymorphic alleles.

5 The present invention may be embodied in other specific forms without departing from its structures, methods, or other essential characteristics as broadly described herein and claimed hereinafter. The described embodiments are to be considered in all respects only as illustrative, and not restrictive. The scope of the invention is, therefore, indicated by the appended claims, rather than by the foregoing
10 description. All changes that come within the meaning and range of equivalency of the claims that follow this specification are to be embraced within their scope.

Claims:

1. A method of detecting the presence of glutathione S-transferase alleles comprising the steps of:

- 5 obtaining a sample of genomic DNA;
amplifying a first portion of the sample with primers for GSTM1 and CDK2;
amplifying a second portion of the sample with primers for GSTT1 and
GSTM3;
amplifying a third portion of the sample with primers for GSTP1; and
10 detecting the alleles and their gene dosage; wherein said detecting shows the
presence or absence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the
gene dosages of GSTM1 and GSTT1.

2. The method of claim 1, wherein at least one primer further comprises a
fluorescent tag selected from the group consisting of TET, FAM(6FAM), 5FAM,
15 TAMRA, HEX, R110, JOE, RG6, NED, and ROX.

3. The method of claim 2, wherein the PCR products of the amplification
of the first, second, and third portions of the sample are combined prior to the step of
detecting the presence and the gene dosage of the alleles.

4. The method of claim 3 wherein the step of detecting the presence or
20 absence of the alleles and their gene dosage comprises conducting a gel
electrophoresis of the sample using a single lane.

5. The method of claim 4, wherein the step of detecting the presence or
absence of the alleles and their gene dosage further comprises fluorescence detection
to determine the presence of PCR products for the specific alleles.
25

6. The method of claim 5, wherein the step of detecting the alleles and their gene dosage further comprises the steps of:

5 comparing the areas under the peak of GSTM1*A or GSTM1*B PCR products with the area under the peak of the CDK2 PCR products to determine the gene dosage of GSTM1; and

comparing the areas under the peak of GSTM3*A or GSTM3*B PCR products with the area under the peak of the GSTT1 PCR products to determine the gene dosage of GSTT1.

10 7. The method of claim 3, wherein the step of detecting the alleles and their gene dosage comprises capillary electrophoresis.

8. The method of claim 7, wherein the step of detecting the alleles and their gene dosage further comprises fluorescence detection to determine the presence of PCR products for the specific alleles.

15 9. The method of claim 8, wherein the step of detecting the alleles and their gene dosage further comprises the steps of:

comparing the areas under the peak of GSTM1*A or GSTM1*B PCR products with the area under the peak of the CDK2 PCR products to determine the gene dosage of GSTM1; and

20 comparing the areas under the peak of GSTM3*A or GSTM3*B PCR products with the area under the peak of the GSTT1 PCR products to determine the gene dosage of GSTT1.

10. The method of claim 2, wherein the primers further comprise internally-biotinylated nucleotides.

11. The method of claim 10, wherein the step of detecting the presence and gene dosage of the alleles comprises the steps of:

combining the PCR products of the amplification of the first, second, and third portions of the sample;

5 exposing the mixture to streptavidin-coated magnetic or non-magnetic beads; and

detecting the presence or absence of allele-specific PCR products through the use of a microplate reader capable of indicating the presence or absence of allele-specific PCR products.

10 12. The method of claim 2, wherein the steps of amplifying a first portion of the sample with primers for GSTM1 and CDK2; amplifying a second portion of the sample with primers for GSTT1 and GSTM3; amplifying a third portion of the sample with primers for GSTP1; and detecting the presence and the gene dosage of the alleles are accomplished using real-time PCR, wherein amplification and detection of the
15 alleles of GSTM1, GSTT1, GSTM3, and GSTP1 and the determination of the gene dosages of GSTM1 and GSTT1 alleles is conducted simultaneously.

13. The method of claim 1, wherein at least one of the primers comprises at least one radiolabeled nucleotide.

14. The method of claim 13, wherein the step of detecting the alleles and
20 their gene dosage comprises detection of the radiolabeled nucleotides.

15. A high-throughput assay for the detection of glutathione S-transferase polymorphic alleles comprising the steps of:

obtaining a sample of genomic DNA;

conducting PCR amplification of the genomic DNA using primers for
25 GSTM1, CDK2, GSTT1, GSTM3, and GSTP1;

combining the PCR reaction products into one mixture; and

detecting the presence and the gene dosage of the alleles.

16. The high-throughput assay of claim 15, wherein at least one of the primers further comprises a label selected from the group consisting of: TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX, biotinylated deoxynucleotides, radioactive phosphorus deoxynucleotides, and radioactive sulfur phosphorothioates.

17. The high-throughput assay of claim 16, wherein the step of detecting the presence and the gene dosage of the alleles comprises loading the mixture into a single gel lane and conducting gel electrophoresis.

18. The high-throughput assay of 16, wherein the step of detecting the presence and the gene dosage of the alleles comprises capillary electrophoresis.

19. A method of identifying the alleles of glutathione S-transferase present in a sample of genetic material, comprising the steps of:

conducting the PCR amplification of the GSTM1 alleles and CDK2 present in a first portion of the sample using primers comprising polynucleotide sequences substantially identical to SEQ ID NO: 1, nucleotides 3–23 of SEQ ID NO: 2, nucleotides 9–31 of SEQ ID NO: 3, SEQ ID NO: 14, and SEQ ID NO: 15; wherein at least one of said primers further comprises a signal marker;

conducting the PCR amplification of the GSTT1 and GSTM3 alleles present in a second portion of the sample using primers comprising polynucleotide sequences substantially identical to nucleotides 7–26 of SEQ ID NO: 4, nucleotides 7–26 of SEQ ID NO: 5, SEQ ID NO: 12, and nucleotides 6–27 of SEQ ID NO: 13; wherein at least one of said primers further comprises a signal marker;

conducting the PCR amplification of the GSTP1 alleles present in a third portion of the sample using primers comprising polynucleotide sequences substantially identical to nucleotides 7–25 of SEQ ID NO: 6, nucleotides 10–28 of SEQ ID NO: 7, nucleotides 6–23 of SEQ ID NO: 8, nucleotides 4–21 of SEQ ID NO: 9, SEQ ID NO: 10, and nucleotides 7–25 of SEQ ID NO: 11; wherein at least one of said primers further comprises a signal marker; and

detecting the presence and gene dosage of the alleles amplified above; wherein said detecting shows the presence or absence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1.

20. The method of claim 19, wherein the signal markers are selected from the group consisting of: TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX, biotinylated deoxynucleotides, radioactive phosphorus deoxynucleotides, and radioactive sulfur phosphorothioates

21. The method of claim 20, wherein the PCR products of the amplification steps are combined prior to the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1.

5 22. The method of claim 21 wherein the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1 comprises analyzing the allele makeup and gene dosage of the sample using a single lane of a gel electrophoresis.

10 23. The method of claim 21, wherein the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1 comprises capillary electrophoresis.

24. The method of claim 21, wherein the primers further comprise internally-biotinylated nucleotides.

15 25. The method of claim 24, wherein the step of detecting the presence and gene dosage of the alleles comprises the steps of:

combining the PCR products of the amplification of the first, second, and third portions of the sample;

exposing the mixture to streptavidin-coated magnetic or non-magnetic beads; and

20 detecting the presence or absence of allele-specific PCR products through the use of a microplate reader capable of indicating the presence or absence of allele-specific PCR products.

26. The method of claim 19, wherein the steps of amplifying a first portion of the sample with primers for GSTM1 and CDK2; amplifying a second portion of the sample with primers for GSTT1 and GSTM3; amplifying a third portion of the sample with primers for GSTP1; and detecting the presence and the gene dosage of the alleles are accomplished using real-time PCR, wherein amplification and detection of the alleles of GSTM1, GSTT1, GSTM3, and GSTP1 and the determination of the gene dosage of GSTM1 and GSTT1 alleles is conducted simultaneously.

27. The method of claim 19, wherein at least one of the signal markers comprises at least one radiolabeled nucleotide.

28. The method of claim 27, wherein the step of detecting the presence of the alleles of GSTM1, GSTM3, GSTT1, and GSTP1, and the gene dosage of GSTM1 and GSTT1 comprises detecting radiolabeled PCT products.

29. A PCR primer comprising a nucleotide sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13.

30. The PCR primer of claim 29, said primer further comprising a signal marker selected from the group consisting of: TET, FAM(6FAM), 5FAM, TAMRA, HEX, R110, JOE, RG6, NED, ROX, biotinylated deoxynucleotides, radioactive phosphorus nucleotides, and radioactive sulfur phosphorothioates.

31. The PCR primer of claim 29, wherein the primer further comprised radiolabeled nucleotides.

32. The PCR primer of claim 29, wherein the primer further comprised internally-biotinylated nucleotides.

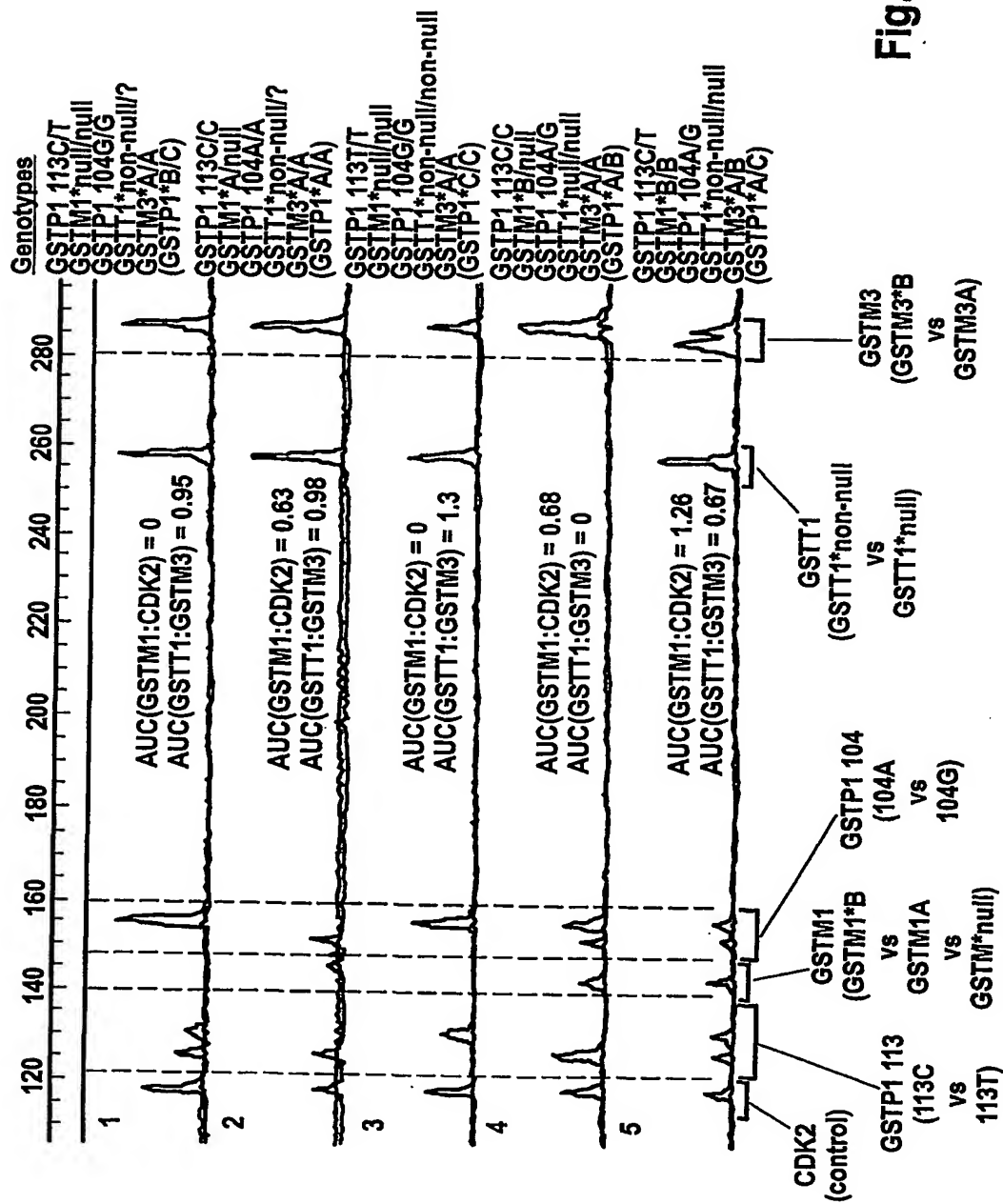


Fig. 1

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Ballard, Linda
Lemons, Richard
Ali-Osman, Francis

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17

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25

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23

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<223> Synthetic oligonucleotide.

<400> 13

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27

<210> 14

<211> 20

<212> DNA

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<220>

<223> Synthetic oligonucleotide.

<400> 14

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20

<210> 15

<211> 21

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<213> Artificial Sequence

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<223> Synthetic oligonucleotide.

<400> 15

aaacttggt tgtaatcagg c

21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/22923

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; C12P 19/34; C07H 21/02, 21/04
 US CL : 435/6, 91.2; 536/23.1, 23.2, 23.5, 24.31, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 435/6, 91.2; 536/23.1, 23.2, 23.5, 24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATTHIAS et al. Cyclin D1, glutathione S-transferase, and cytochrome P450 genotypes and outcome in patients with upper aerodigestive tract cancers: assessment of the	1-32
Y	ISHII et al. Glutathione S-transferase P1 (GSTP1) polymorphism in patients with chronic obstructive pulmonary disease. Thorax. 1999, Vol. 54, pages 693-696, especially pages	1-32
Y	OLSHAN et al. GSTM1, GSTT1, CYP1A1, and NAT1 polymorphisms, tobacco use and the risk of head and neck cancer. Cancer Epidemiology, Biomarkers and Prevention.	1-32
Y	KERB et al. Detection of the GSTM1*0 allele by long polymerase chain reaction. Pharmacogenetics. 1999, Vol. 9, pages 89-94, especially pages 90-91.	1-32
Y	MATTHIAS et al. Polymorphism in cytochrome P450 CYP2D6, CYP1A1, CYP2E1 and glutathione S-transferase, GSTM1, GSTM3, GSTT1 and susceptibility to tobacco-related	1-32
Y	JOURENKOVA-MIRONOVA et al. Glutathione S-transferase GSTM1, GSTM3, GSTP1 and GSTT1 genotypes and the risk of smoking-related oral and pharyngeal cancers.	1-32
Y	NEDELEHEVA et al. Single tube multiplex polymerase chain reaction genotype analysis of GSTM1, GSTT1 and GSTP1: relation of genotypes to TP53 tumor status and	1-32
Y	TO-FIGUERAS et al. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) polymorphisms and lung cancer risk among Northwestern Mediterraneans.	1-32
Y	AHMADI et al. GSTM1 and mEPHX polymorphisms in Parkinson's disease and age of onset. Biochemical and Biophysical Research Communications. 2000, Vol. 269, pages 676-680, especially page 677.	1-32

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 27 September 2001 (27.09.2001)	Date of mailing of the international search report 19 NOV 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer C. Myers Telephone No. 703-308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/22923

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,965,363 A (MONFORTE et al) 12 October 1999, columns 14-16	1-28
Y	US 5,888,778 A (SHUBER, A.P.) 30 March 1999, columns 8-10	1-28
Y	DE 19738908 A1 (PFENNING MEINIG & PARTNER GbR) 11 March 1999, Figure 1.	29-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/22923

Continuation of B. FIELDS SEARCHED Item 3:

DIALOG: MEDLINE, CA, BIOSIS, EMBASE, SCISEARCH; WEST-US, EP, JP, WO PATENTS

search terms: genomic, quantitative, pcr, amplification, gene dosage, copy number, heterozygous, homozygous, GSTM1, CDK2, GSTT1, GSTM3, GSTP1